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Factors affecting the formation of bile canaliculi.

by

Timothy Lee Terry ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

Edmonton, Alberta
Fall 1994

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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate studies and Research for acceptance, a thesis entitled

Factors affecting the formation of bile canaliculi.

submitted by Timothy Lee Terry
in partial fulfilment of the requirements for the degree of
MASTER OF SCIENCE

(Dr. W.J. Gallin, Supervisor)

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Date: July 22/94

Abstract

The polarization of hepatocytes to form a connected network of bile canaliculi (BC) is necessary for the function of the liver. Hepatocyte polarization may be controlled by soluble factors and/or physical interactions between cells. Monolayer cultures of embryonic chicken hepatocytes in DMEM supplemented with ornithine, dexamethasone, and insulin express BC-specific antigens for at least 7 days. However, BC-specific antigen expression is lost within 3 days of culture initiation in DMEM containing 10% fetal calf serum. The dedifferentiating effects of FCS can be reversed. Furthermore, cultures in medium containing ornithine, dexamethasone, insulin and 10% FCS appear identical to cultures grown in 10% FCS alone. Thus FCS contains a soluble inhibitor of hepatocyte polarization. Aggregate cultures grown in suspension maintain hepatocyte polarization for 10-12 days. This may be due to the increased cell-cell contact between hepatocytes in aggregate culture, or to more normal contact with the extracellular matrix (ECM). I have evaluated the role of cadherin-mediated interactions on hepatocyte polarization. Anti-E-cadherin Fab' fragments disrupted the formation of long networks of BC in monolayer cultures but did not stop polarized expression of BC-specific antigens. The BC antigens in anti-E-cadherin-treated cells were concentrated in small areas between cells and were present at lower levels uniformly on the cell surface. These results

indicate that E-cadherin is required for the formation of extended BC networks, but that other factors are responsible for maintaining the synthesis and localization of BC-specific antigens.

Preface

The liver has been studied since ancient times. Early investigators were interested in the blood supply and histology of the liver, and formulated hypotheses as to the liver's function. One such observer was Herophilus in Alexandria, who may have performed some of the first public dissections of human bodies in 300 BC. He was especially interested in the eye and described the brain as the centre of the nervous system. He also worked on the digestive tract (naming the duodenum) and showed that the liver had a different shape in different individuals. This work on the liver supported previous beliefs on the regenerative capacity of the liver that originated in antiquity. The ancient Greeks included the regenerative abilities of the liver in their mythology. Prometheus the Titan, who appeared in some of the earliest Greek literature, stole fire for the people of earth and as a punishment for this sin was sentenced by Zeus to spend eternity having an eagle pick out his liver by day while at night the liver regenerated to provide fresh fodder for the eagle in the next day.

Today, investigators are interested in the metabolism, morphology, and cell physiology of the liver. In order to do experiments which address these issues, a long-term culture method providing hepatocytes that maintain their differentiated phenotype is needed. Considerable advancement has been made in liver cell culture methods;

however, most liver cell cultures de-differentiate within 3 days of culture initiation. The reasons for this de-differentiation are currently unclear.

This thesis describes the development of culture techniques used to support the formation and maintenance of bile canaliculi in embryonic chicken liver. Furthermore, while investigating the importance of cell-cell interactions on the formation and maintenance of bile canaliculi, I have found that E-cadherin mediated cell-cell adhesion is important for the formation of long range networks of bile canaliculi. However, the hepatocytes are able to maintain a polarized distribution of bile canaliculi-specific antigens in anti-E-cadherin treated cultures. Thus, some factor other than E-cadherin is responsible for the maintenance of the polarized distribution of these antigens.

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ABBREVIATIONS

BC:	bile canaliculi
BSA:	bovine serum albumin
D:	Dulbecco's Minimal Essential Medium with Pen/Strep
DB:	Dulbecco's Minimal Essential Medium with Pen/Strep, 10 μM dexamethasone, $0.2X10^3$ U/ml insulin, 67.4 mg/L ornithine, and 0.5% fatty acid free BSA
DIOD:	Dulbecco's Minimal Essential Medium with Pen/Strep, $0.2X10^3$ U/ml insulin, 67.4 mg/L ornithine, and 10 μM dexamethasone
DF:	Dulbecco's Minimal Essential Medium with 10% fetal calf serum, and 1μg/ml gentamicin
DMSO:	Dimethyl sulfoxide
ECM:	extracelluar matrix
ER:	endoplasmic reticulum
FCS:	fetal calf serum
FGF:	fibroblast growth factor
GAGs	glycosaminoglycans
HBGF-1	heparin-binding growth factor type 1
HGF:	human growth factor
PB:	phosphate buffer
SV40	Simian virus 40
TEM:	transmission electron microscope
TGF:	transforming growth factor

ZO-1: zonula occludens-1

ZO-2: Zonula occludens-2

CHAPTER I

Introduction

Function

The liver performs a number of essential metabolic functions, including regulating blood glucose levels, producing all plasma proteins except the immunoglobulins, and detoxifing endogenous and exogenous compounds (Guguen-Guillouzo, 1992). The liver also produces bile which in most animals is stored in the gall bladder and released into the small intestine. Two of the major functions of bile are the removal of conjugated wastes from hepatocytes and the transport of bile salts to the small intestine in which they are used to emulsify lipids making them vulnerable to lipases. Therefore the liver acts as an interface between the circulatory system and the digestive system, utilizing a system of tubules which begin at the hepatocytes.

The liver's main functional cell is the hepatocyte. Mature hepatocytes have similar morphologies including a basal nucleus with one or two nucleoli, cisternae of the rough ER (endoplasmic reticulum) bordering the plasmalemma, little smooth ER, and mitochondria situated in the cell apex (Wong & Cavey, 1992).

The spatial arrangement of the hepatocytes in relation to the other cell types in the liver enable them to perform their specialized functions. The hepatocytes are separated

from the liver sinusoids by the space of Disse (perisinusoidal space) which is virtually collagen- and fibronectin-free (Figure I-1). Blood plasma, which percolates between the endothelial cells lining the sinusoids, crosses the space of Disse. Macromolecules in the plasma can be internalized by receptors on the basal surface of the hepatocyte. Once inside the hepatocyte this material is processed and the end products may be secreted into a system of tubules at the apical surface of the hepatocyte and transported into the small intestine.

Bile canaliculi (BC) are formed at the apical surfaces of hepatocytes and are the smallest set of tubules in the path utilized to move metabolic waste and bile salts from the hepatocyte to the lumen of the small intestine. From the BC the bile flows into the portal canals, then into the bile ductules (or Hering's ducts), the bile ducts, and finally into the hepatic ducts which subsequently leave the liver.

The formation of BC and directional secretion of materials into the canaliculi requires the establishment of epithelial cell polarity which correlates with the formation of specialized cell-cell junctions and striking changes in the organization of the cytoskeleton (Wacker et al., 1992).

There are several cytoskeletal components in associated with BC. These include actin filaments in the microvilli,

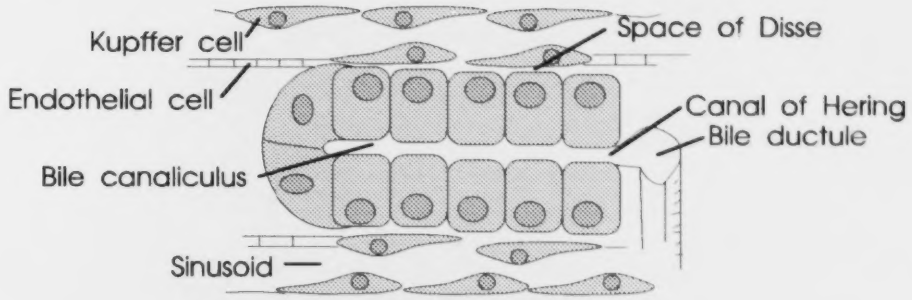

Figure I-1 Schematic diagram of chick liver bile canaliculi and associated cells

cytokeratins in the pericanalicular sheath, and microtubules in the hepatocyte cytoplasm (Kawahara, et al., 1989). Kawahara et al. (1990) performed a series of experiments on monolayers of rat hepatocytes in which they evaluated the ability of hepatocytes exposed to cytochalasin B (depolymerizes F-actin), colchicine (depolymerizes microtubules), and nickel (rearranges cytokeratin filaments) to take up, transport, and secrete into BC, markers such as fluorescein diacetate and horseradish peroxidase. They demonstrated that microtubules play a role in the secretion of materials into the BC, microfilaments play a role in canalicular contraction, and cytokeratin intermediate filaments have a role in the formation of BC by providing a structural scaffolding, allowing uptake of materials via

endocytosis into hepatocytes, and assist the secretion of passively internalized material into BC (Kawahara and French, 1990; Kawahara, et al., 1989; Kawahara et al., 1990).

Intercellular adhesion, the formation of junctional complexes, and the formation of a polarized distribution of surface components are required for the transport of materials across an epithelium (Wollner, et al., 1992). Junctional complexes include tight junctions, adherens junctions, desmosomes, and gap junctions. Tight junctions are the most apical components of a junctional complex and restrict the free passage of molecules and ions transcellularly across an epithelium. Tight junctions also allow epithelia to form apical and a basolateral plasma membrane domains and prevent mixing of components localized in either domain, allowing an epithelium to carry out its specialized functions (reviewed in (Rodriguez-Boulan & Nelson, 1989)). However, the precise role tight junctions play in preventing the free movement of proteins and lipids into inappropriate membrane domains is unclear since neurons, which lack intercellular tight junctions, still have a polarized lipid distribution (Kobayashi, et al., 1992). The cytoplasmic plaque of tight junctions has several components which include ZO-1 (Stevenson, et al., 1986), ZO-2 (Gumbiner, et al., 1991), and usually cingulin (Citi, et al., 1988)(not in chick kidney glomeruli (Citi, et

al., 1989)). F-actin is also associated with tight junctions cytoplasmically and, as shown by studies with cytochalasin, plays a role in the maintenance of tight junctions (reviewed in (Madara, 1992)). It is not known if any of the proteins associated with tight junctions interact with F-actin directly or indirectly through actin-binding proteins.

Desmosomes are used for cell-cell adhesion and provide attachment sites for cytokeratin intermediate filaments (Steinberg et al., 1987). By linking intermediate filament networks, desmosomes may confer structural continuity and tensile strength to epithelia and transmit signals between the cytoskeletons of adjacent cells. Desmosomes are associated with both integral membrane components and peripheral components. The integral membrane components include the N-cadherin-like (Collins, et al., 1991) desmoglein 1 and desmocollin 1 and 2 (Crowin & Garrod, 1983; Parker, et al., 1991), and several other polypeptides. The plaque components include desmoplakin 1 and 2, plakoglobin, desmocalmin (Crowin & Garrod, 1983; Mueller and Franke, 1983; Kapprell, et al., 1985), keratocalmin, and several other proteins which may play a role in binding the cadherin-like membranous proteins to the cytokeratin filaments. Desmosomes are 0.5-2.0 μm in diameter and are approximately 100 nm thick. The intercellular space between desmosomal plaques is 20-30 nm and contains the extracelluar

domains of the transmembrane glycoproteins of the desmosome (Stappenbeck and Green, 1992).

Adherens (intermediate) junctions form a belt of cell-cell adhesion between epithelial cells. The motility of actin filaments associated with adherens junctions may be involved in the folding of epithelial sheets during morphogenesis, and in the transmission of forces and morphogenetic signals between the cytoskeletons of adjacent cells. Structurally, these junctions consist of a dense plaque on the cytoplasmic side of the plasma membrane and an intercellular 15-20 nm gap. E-cadherin or N-cadherin is found spanning the membrane and joins adjacent cells. The cytoplasmic plaque contains proteins such as catenins (Nagafuchi et al., 1991) which may connect cadherins to the cytoskeleton, and several actin binding proteins including myosin, tropomyosin, alpha actinin, and vinculin (Tsukita & Tsukita, 1989). These may bind cadherins to the cytoskeleton directly or may do so by forming a protein complex. The expression of these components changes over time, possibly correlating with the importance of these facilitators in cellular adhesion (Stamatoglou et al., 1992) or in signal transduction, since β-catenin is homologous to the *Drosophila* signalling protein armadillo.

Gap junctions are also present on the lateral surface of cells. However, unlike the other components of the junctional complex they are not involved in cellular

adhesion but instead are used for the direct exchange of small molecules between the cytoplasm of adjacent epithelial cells. Gap junctions are composed of several connexons, each of which is composed of six connexin subunits. The two major connexin subunits in the liver are connexin 32 and connexin 26 (Nicholson, et al., 1987), while connexin 43 is undetectable in differentiated hepatocytes. In the centre of the connexon is a pore which permits intercellular passage of materials under 1200 daltons such as inorganic ions and cyclic AMP. Thus, these junctions may be involved in co-ordinating cellular events such as the regulation of cell division and differentiation.

The model

Early investigators chose chickens for the study of liver development because early organogenesis of the liver is difficult to study in a mammalian system. In mammals the embryonic liver is a major erythropoietic organ, making hepatic cells difficult to observe through the erythropoietic precursor cells. Study of the chicken embryo reduces this problem because initial blood formation takes place in the yolk sac. The experiments outlined in this thesis were done with 14-day embryonic chicken livers and, since the cells were excised and cultured, the presence of erythropoietic cells was not a primary concern. In addition, chick tissue is easily available and a large

library of reagents whose activities have been previously characterized against embryonic chicken cells was already available in our laboratory.

This thesis describes some of the conditions necessary for the formation and maintenance of BC in primary cell culture. However, to understand how BC form in culture, one must first understand how they form *in vivo*.

Chick liver formation *in vivo*

Early development

The first liver precursor, the cranial hepatic diverticulum, arises in the second day of incubation (16 somite embryo (Hamburger and Hamilton (1951) stage 12)) as an evagination of the foregut, dorsal to the paired omphalomesenteric (vitelline) veins just before they enter the sinus venosus (Romanoff, 1960)(Figure I-2). This diverticulum grows anteriorly, dorsal to the ductus venosus, and into the septum transversum. A second evagination, the caudal hepatic diverticulum, arises in the chick at the 19 somite embryo (stage 13) from the floor of the foregut. It is posterior to the first evagination and ventral to the ductus venosus. It then extends between the omphalomesenteric veins and anteriorly towards the septum transversum (Romanoff, 1960)(Figure I-3).

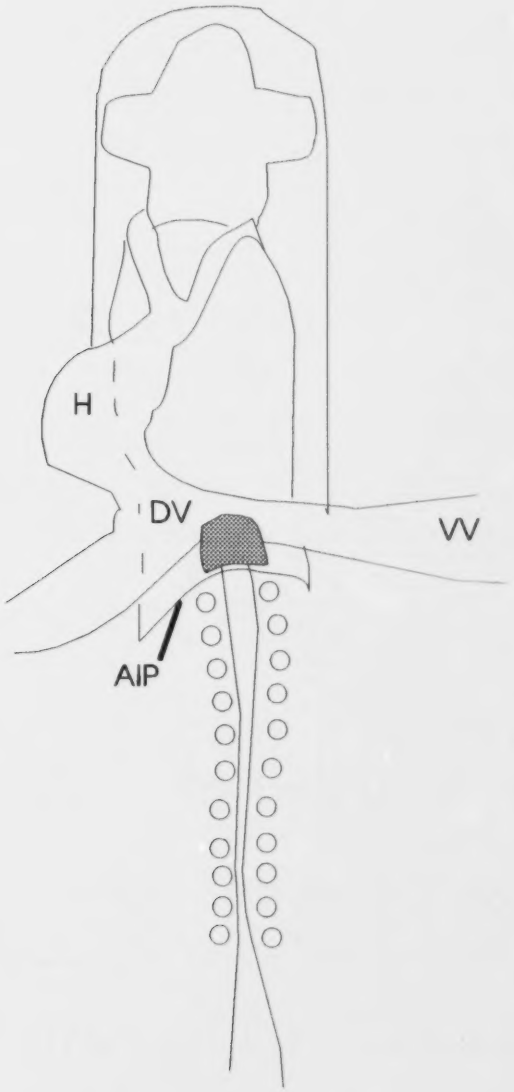

Figure I-2 Stage 12 chicken embryo showing the hepatic primordium (shaded), anterior intestinal portal (AIP), ductus venosus (DV), heart (H), and the vitelline vein (VV). Adapted from Fukuda, 1976.

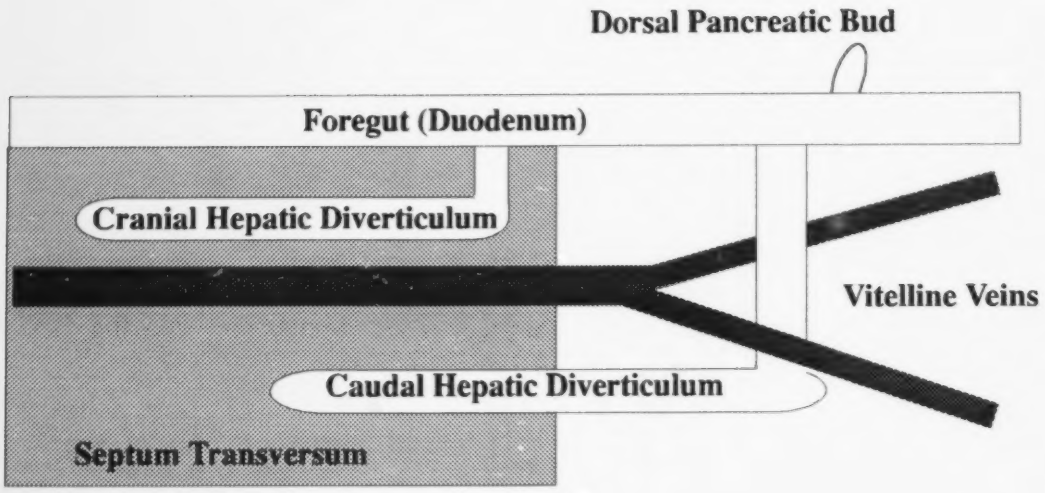

Figure I-3 Early hepatic diverticula invading the septum transversum.

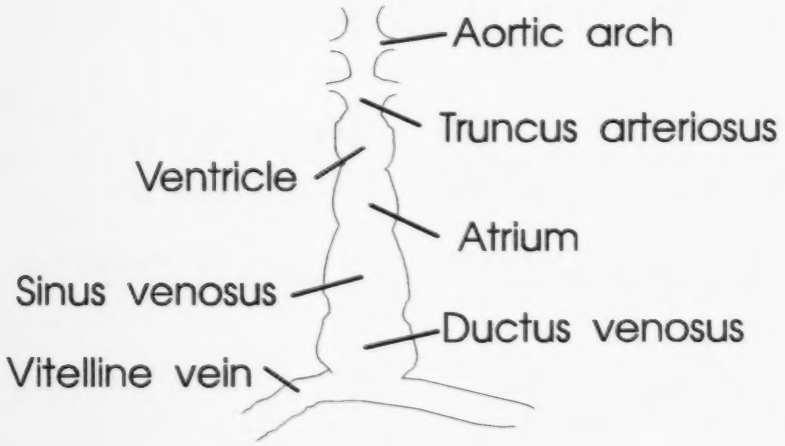

Figure I-4 Regions of the developing heart and associated blood vessels.

At approximately 50 hours of incubation (stage 14) the caudal hepatic diverticulum branches into right and left buds. By 60 hours (stage 16) the flattened buds of the caudal diverticulum have expanded and the endodermal gutter between the diverticula has deepened into the hepatic furrow (Kingsbury et al., 1956). During the third day there appears a proliferation of buds from the cranial hepatic diverticulum. Late in the third day the caudal end of the hepatic furrow forms an epithelial thickening which later forms the gall bladder (Kingsbury et al., 1956).

Early in the fourth day (stages 18, 19), buds from the cranial hepatic diverticulum elongate to the cephalic end of the ductus venosus (Kingsbury et al., 1956). These buds branch mostly left around the ductus venosus, while buds from the caudal hepatic diverticulum branch to the right. Together they form a mass of hepatic cords encircling the ductus venosus, some of which contain lumina (Kingsbury et al., 1956), and sinusoids (Fukuda, 1976; Fukuda and Mizuno, 1978; Fukuda, 1979). Thus, on the fourth day of chicken development the hepatocytes begin to differentiate from the foregut endothelium and form BC, while the sinusoids form from the ductus venosus (Le Douarin, 1975; Fukuda, 1976; Fukuda, 1979). In the region of the anastomosed cords the luminated buds from the original diverticula fuse and form the connection between the future gall bladder and the portion of the liver arising from the cranial hepatic

diverticulum (Kingsbury et al., 1956). Thus the distal portions of the hepatic diverticulum form the liver while more proximal portions form the biliary ducts and gall bladder (Romanoff, 1960). The septum transversum ostensibly provides the hepatic connective tissue, hemopoietic stem cells, and Kupffer cells for the hepatic sinusoids (Rifkind et al., 1969). The expansion of the liver is limited anteriorly by the heart and the septum transversum, and to the left by the stomach (Kingsbury et al., 1956). During the fourth day of incubation a lateral curvature develops in the stomach which creates room for enlargement of the liver.

On the fifth day the remaining solid cords of hepatic trabeculae develop lumina, increasing the number of thick walled tubes which open into the primary diverticula (Lillie, 1952). Eventually the proliferation and anastomoses of the hepatic cylinders result in a pattern of cellular masses forming thin hepatic cords from the original thick cords.

The liver enlarges during days 5 and 6 (stages 22-27). The anterior portion expands into the coelomic space made available by the absorption of the sinus venosus into the right atrium (Kingsbury et al., 1956). Concurrently, the central portion of the liver bulges to the right and a small extension forms to the left and ventral to the stomach (Kingsbury et al., 1956). This division into left and right lobes is marked by two major ligaments, the dorsal

gastrohepatic and the ventral falciform ligament (Kingsbury et al., 1956).

On day 7 (stage 30) the liver has reached its furthest extent within the septum transversum, invaded the allantoic veins, and encased the inferior vena cava (Kingsbury et al., 1956). Then during the eighth and ninth days of incubation (stages 31-35) there is an increase in the number of cords and a decrease in the size of the sinusoids, making the liver more compact (Kingsbury et al., 1956). The divisions of the portal and hepatic veins are instituted during days 10-14 (stages 36-40)(Romanoff, 1960). On days 15-21 (stages 41-46) there is a growth in hepatic parenchyma and the sinusoids are constricted and more slit-like (Kingsbury et al., 1956). The venous pattern is set and there are more interior veins present. There is also a reduction in the number of hepatocytes arranged radially around each BC. In the early cords (Stages 17 and 18), 6-13 hepatocytes ring each canaliculus (Fukuda, 1976), and the cell number tends to normalize at 6 by stage 41 (Kingsbury et al., 1956).

Basic histology of the chick liver

Mammal and chick liver differ considerably in structure. The mammalian liver is formed by a network of anastomosing plates which are 1-2 cell layers thick; the BC are found between the cells of the plates (Elias, 1949a; Elias, 1949b; Hodges, 1972). Mammalian hepatic plates are

arranged radially around central veins and form lobules (Elias, 1949a; Elias, 1949b). In chicken liver there is a mass of branching, hollow cords, and the BC are found in the centre of the cords (Romanoff, 1960). Furthermore, there is little evidence of lobulization in the chick liver (Hodges, 1972; Romanoff, 1960) due to the lack of interlobular septa (Gerber & Thung, 1987). Hepatic sinusoids of a mammalian embryo are smaller than those found in birds, possibly due to the presence of extra-vascular clusters of hemopoietic cells in the mammal (Le Douarin, 1975). In both mammals and birds the discontinuous linings of the sinusoids consist of endothelial cells and Kupffer cells (Bankston & Pino, 1980; Hodges, 1972)(Figure I-1). The sinusoidal lumenae and the perisinusoidal spaces may be linked directly by fenestrae traversing the endothelial cells or by intercellular gaps in the sinusoidal linings.

In most birds the liver consists of a left lobe ventral to the proventriculus and gizzard, and a larger right lobe ventral to the pancreas, the open end of the duodenal loop, and part of the ilium (Romanoff, 1960). There are two ducts that carry bile from the lobes into the duodenum. One duct is the hepatic or hepatoenteric duct, which arises from the left lobe. The other duct arises from the right lobe and has the gall bladder inserted into it, thus dividing into the hepatocystic and a cystoenteric or cystic duct (Romanoff, 1960). Some avian families are missing the gall

bladder; these are the Psittacidae, Cuculidae, and Columbidae.

Historical summary of cell culture

Hepatocytes are the major functional cell type of the liver; therefore these cells are of interest to investigators examining liver function. The cells are used as a simple model for studying liver diseases and as a tool for measuring the hepatotoxicity and metabolism of drugs, biosynthesis of proteins, and gene regulation (Tong et al., 1992). To carry out these studies the cells must first be successfully cultured and this relies on a large body of previous work.

A major problem with the formation of successful cell cultures was finding conditions which maintained the cells alive over many generations. Wilhelm Roux, in 1885, was one of the first investigators to culture cells outside the body. He explanted pieces of chick embryo into warm saline and observed the cells over a period of several days (Sharp, 1977). In 1907 Ross Granville Harrison, who was interested in the development of the peripheral nervous system, observed that pieces of a frog embryo would continue to grow and differentiate if they were cultured with lymph from an adult frog. However, even with the extreme measures Harrison devised for maintaining sterile conditions these cultures would always die within a few weeks of culture

initiation. Although Harrison was not the first to culture cells outside of the body and was not exceedingly successful with his cultures, he is still considered the father of tissue culture because he showed that adaptation of the hanging drop technique used by bacteriologists could be used to keep tissue alive for several weeks and that the procedure was a research method capable of making significant scientific progress possible (Sharp, 1977).

M.T. Burrows was the first to present a major improvement over Harrison's technique. The frog lymph used in Harrison's technique did not solidify, nor was it easy to obtain in sufficient quantities for large-scale experiments; therefore, Burrow used chicken plasma to support and nourish explants of chick embryonic tissue in hanging drops. Chicken plasma proved to be superior to frog lymph in that the plasma provided good growth of the nervous tissue, heart, and the skin (Sharp, 1977). Then, using similar techniques, Burrows and Alexis Carrel succeeded in growing explants from adult dogs, cats, rats, and guinea pigs. In addition they showed that the lifespan of cells *in vitro* could be extended by subculturing the cells into fresh media. They also found that by mixing a portion of chicken embryo extract with chick plasma there was greatly improved cell survival and growth (Sharp, 1977). Then, in 1912, Carrel proved that cells were not intrinsically unstable and that, if fed, they would continue to grow indefinitely in

culture.

The length of time in which a culture remained viable was still largely determined by the time required for microbial contamination. Thus the discovery of antibiotics in the 1940's allowed large-scale, long-term culturing of cells.

While most investigators were trying to maximize cell growth in undefined media, W.H. Lewis and M.R. Lewis were replacing the plasma/embryo extract medium in common use, which had an unknown composition, with a synthetic media composed of known components. Other investigators furthered this work over the next several decades. Eagle, in 1955, in the first systematic investigation of the essential nutrients required of cells in tissue culture, found that HeLa and the mouse L-cell line needed only 1% of dialysed horse serum in an otherwise defined media for growth. Then in 1958, Fisher et al. showed that fetuin (a major protein in fetal bovine serum and associated with some growth factors)could substitute for fetal calf serum to promote the spreading of cells onto glass, perhaps by inhibiting proteolysis on the cell surface.

In 1962 Eagle and Piez showed that HeLa and other cell lines had a requirement for certain essential nutrients such as serine. Then in 1965 Ham introduced a serum-free defined medium for the culture of mammalian cells (Ham, 1978), while in 1976 Sato showed that different types of cells require

different mixtures of hormones and growth factors to grow in a serum-free medium.

Summary of liver culture techniques to date

Attempts have been made to generate pure hepatocyte populations; however, the short-term survival and poor differentiation of the isolated cells proved limiting. Investigators have tried to determine what factors are responsible for maintaining the function of differentiated hepatocytes and have found that a mixture of nutritive factors and other factors such as cell-cell contact and biomatrix deposition may be required (Guguen-Guillouzo, 1992).

Although many of the factors required for the formation and maintenance of differentiated cells are known, only transient cultures of mature hepatocytes have been attained. The transiency of the cultures is due to our incomplete understanding of factors which control hepatocyte differentiation. Tissue differentiation such as that seen in hepatocytes is regulated by hormones, growth factors, intercellular communication, and by communication between cells and their extracelluar matrix (Stoker et al. 1990; Wollner et al., 1992; Saxen et al. 1968; Ekblom, 1989). Investigators have tried to replicate the presence of insoluble compounds and the matrix components of the hepatic sinusoids, the trabecular organization of hepatocytes which

are closely surrounded by the sinusoidal cells, and the direct contacts of hepatocytes with the epithelial cells of the canal of Hering, by co-culturing hepatocytes with other types of liver cells. Considerable progress was made using untransformed 10-day rat liver cells but the addition of corticosteroids to the medium was needed to replicate the co-culture effect. Thus some soluble factor is needed in this model (Guguen-Guillouzo and Guillouzo, 1983; Guguen-Guillouzo et al., 1984).

Hepatocytes have been cultured under conditions in which spheroid (aggregate) formation is favoured. These hepatocytes have characteristics similar to those found in liver tissue (Tong et al., 1992). This indicates that the normal regulatory processes which occur *in vivo* may be hampered by the two-dimensional monolayer culture methods often associated with cell culture (Landry et al., 1985). Thus rat liver cell aggregates may be able to produce the minimal essential requirements needed for long-term maintenance of the culture, even in the absence of added serum or hormones (Landry and Freyer, 1984; Landry et al., 1985).

Co-culturing hepatocytes with other cell types provides a means of generating stable long-term hepatocyte cultures and gives the capacity to mimic the complex environment found *in vivo*. The first attempts at co-culture of hepatocytes were with sinusoidal cells (Wanson et al., 1979)

or human fibroblasts (Michalopoulos, 1990). These cultures were unsuccessful, showing only minor improvements in liver function. Much more progress was made using untransformed epithelial cells, probably derived from primitive biliary cells (Guguen-Guillouzo and Guillouzo, 1983; Clement et al., 1984). Later investigators began to try permanent cell lines for co-culture because of their stable phenotype and ease of growth (Donato et al., 1990). One such cell line is the rat liver epithelial cells (RLEC) cell line. When co-cultured with RLEC, hepatocytes from various species survive for several weeks and retain some liver-specific functions (Guguen-Guillouzo et al. 1984). In contrast to the functioning of hepatocytes in a serum-free, defined medium, hepatocytes co-cultured with RLEC retain their ability to transcribe specific genes at a rate similar to that seen in DMSO treated cells or in cells grown in Matrigel (Fraslin et al., 1985). Other cell lines used include hepatoma cell lines of tumour origin which constitutively express specific markers (Ihrke, et al., 1993; Sormunen, et al., 1993). However, transformation is associated with a variety of morphological and functional changes which coincide with an alteration of gene regulation. Furthermore, no hepatoma cell line expresses all of the functions exhibited by untransformed hepatocytes (Guguen-Guillouzo, 1992).

Perhaps the most promising area of research is in the production of immortalized nontransformed cell lines. Paul

et al. (1988) established non-tumorigenic hepatocytes from the liver of a transgenic mouse. The mouse has a transgene consisting of the large tumour antigen (TAg) from SV40 (Simian virus 40) under the control of the liver-specific mouse metallothionein I promoter. These cells were found to be highly differentiated, expressing many liver-specific genes, and stop dividing in confluent cultures using a mitogen-free medium. Furthermore fusions of rat and human cells have been used to study polarization. Cassio et al., in 1991, used WIF12-1 cells which are formed from hybrids between Fao rat hepatoma cells (Deschatrette and Weiss, 1974)and WI 38 human fibroblasts and found that these cells expressed liver-specific functions at high levels. The cells generate a polarized distribution of apical markers such as Mg^{++}-ATPase and form BC which rhythmically expand and contract (Cassio,et al., 1991).

Culture conditions found to encourage hepatocyte polarization

Traditionally, there has been a problem of de-differentiation of epithelial cells. In most of the early attempts at epithelial cell culture the cause of the dedifferentiation was an overgrowth of the culture by cell types other than those of primary interest, such as stromal fibroblasts or undifferentiated cells. This problem can be solved through selection of the cell type of interest with

various cell separation methods including selective attachment, selective detachment, the use of feeder layers, or selective culture media.

Another problem that must be addressed is the induction of cell differentiation. Culture conditions may select for cell proliferation or for cell differentiation (Freshney, 1992). Thus, the loss of differentiated traits in an epithelial cell culture results from overgrowth of the culture by un-differentiated cells. A change in culture conditions could cause these un-differentiated cells to differentiate.

The induction of differentiation appears to be regulated by four inter-dependent factors. These factors are soluble inducers, cell-cell interactions, cell-matrix interactions, and polarity and cell shape. These factors may be permissive or directly inductive (Freshney, 1992).

Many cell-cell interactions require cellular adherence. When placed into a culture under basal conditions the isolated hepatocytes adhere to each other. This indicates that they retain the cell surface components required for homotypic adhesion. The hepatocytes reaggregate quickly and associate with the substrate via negative charges, and/or through binding to ECM materials, to reconstitute the trabeculae formed *in vivo*. Initially the cells are able to perform most of the functions they would perform in the body as tested through the production of specific enzymes such as

Mg^{++}-ATPase, a histochemical marker for canalicular membranes, or other plasma membrane proteins. These properties are reduced within the first 3 days of culture (Gallin & Sanders, 1992; Guguen-Guillouzo and Guillouzo, 1983; Sirica et al., 1979; Steward et al., 1985). Thus liver-specific gene transcription may be blocked once the cells are isolated (Clayton and Darnell, 1983).

Soluble factors play a role in the differentiation of hepatocytes and may be used to improve cellular functions and/or to induce DNA synthesis *in vitro* (McGowan, 1986). Soluble factors include the hormone hydrocortisone (Speirs and Freshney, 1991), vitamins such as retinoic acid (Klann and Marchok, 1982; Wu and Wu, 1986), paracrine factors (some of which seem related to FGF)(Rudland et al., 1982), and prostaglandins (Freshney, 1992), and planar-polar compounds such as DMSO (dimethyl sulfoxide)(Rudland, et al. 1982), hexamethylenebisacetamide, sodium butyrate, and N-,methyl- and dimethylformamide (Dexter et al., 1979).

DMSO is an important culture additive and no other soluble factor has been found which, alone, will maintain hepatocyte differentiation for more than a few days. When added at the final concentration of 1.5-2% to a serum-free medium, DMSO prolongs hepatocyte survival and preserves cellular functions for at least one month (Isom et al., 1985; Isom et al., 1987). There is a preservation of most of the liver-specific functions; the cells remain cuboidal,

form BC, and do not divide. The use of DMSO is useful for genetic studies but is questioned for other types of work because the solvent is used at a high concentration and is clearly not a factor naturally associated with cells.

Corticosteroids are soluble factors that have a dose-dependent beneficial role in hepatocyte survival and function *in vitro* (Guguen-Guillouzo, 1992). Dexamethasone is an effective corticosteroid and often used at 10^{-6} M. At this concentration it greatly improves viability, the formation of BC, and the performance of hepatocyte-specific functions (Guguen-Guillouzo, 1992). Insulin (10^{-7}-10^{-6} M) has also been found to be beneficial but not essential in increasing hepatocyte attachment, inducing total *de novo* protein synthesis, and regulating many liver-specific functions such as metabolism of lipids and carbohydrates from the diet (Agius et al., 1990). However, in the presence of glucocorticoids and insulin, the transcription of many liver-specific genes decreased to 3-10% of the *in vivo* level within 24 hours of culture initiation, while the total mRNA levels stay the same as those found *in vivo* which implies that these hormones may be involved mainly in mRNA stabilization (Jefferson et al., 1984).

Two types of soluble factors used in hepatocyte culture are humoral factors and intermediary metabolites. There are two main groups of humoral growth promoting agents, the primary mitogenic agents and the co-mitogenic factors.

Mitogenic agents include EGF (epidermal growth factor) (McGowan, 1986), TGF-α, HBGF-1 (heparin-binding growth factor type 1) of a FGF (fibroblast growth factor) (Kahn et al., 1989), and Hepatoprotein A or HGF (human growth factor) (Nakamura et al., 1988). Co-mitogenic factors include norepinephrin (Michalopoulos, 1990), vasopressin (Russell and Bucher, 1983), estrogens, androgens II and III, insulin (McGowan, 1986), and glucagon. Mitogenic factors stimulate cells to enter S phase of the cell cycle; however, maximal stimulation of DNA synthesis is achieved when they are used in conjunction with co-mitogenic factors (Michalopoulos, 1990). Intermediary metabolites such as pyruvate or lactate are also important in promoting hepatocyte DNA synthesis and the production of specific proteins such as albumin (McGowan and Bucher, 1983).

Salts and electrolytes prolong hepatocyte survival from 5 days to 10-12 days. This effect may be accomplished by decreasing the bicarbonate concentration of the medium to 5 mM or by increasing the osmolarity of the medium by adding 50-100 mM sodium chloride (McGowan, 1986). The differentiation of some epithelia is sensitive to the concentration of Ca^{2+} (Boyce and Ham, 1983), with concentrations above 3 mM causing differentiation and lower concentrations favouring cell proliferation.

In addition to cell-cell contact and soluble factors the plating density of hepatocytes, as in other cells,

influences the maintenance of differentiated hepatocyte functions. Low plating densities result in hepatocyte cultures which are more responsive to growth factors than hepatocytes grown at higher densities (Nakamura et al., 1983; Guguen-Guillouzo, 1986). However, a high cell density may be necessary for intercellular communication via gap junctions which allow the transfer of signals, such as cyclic AMP. These signals are needed to decrease the rate of cell proliferation or to change the shape of the cells due to crowding.

An appropriate cell density may optimize the interactions between epithelial cells and mesenchymal cells needed for embryonic organogenesis (Thesleff et al., 1977; Wessells, 1977; Rutter et al., 1978). Formation of hepatic cords requires both liver mesenchyme and sinusoidal epithelium (Sherer, 1975). Epithelial-mesenchymal interactions are very specific; the mesenchyme determines the fate of the epithelium. Thus it is likely that at least part of a culture's failure to maintain polarization or to generate polarized cells is due to a failure of the epithelial cells to interact correctly with the stromal cells. Stromal cells may participate in the induction of differentiation through interactions with steroids. For example, prostatic differentiation is under androgen control, but the actions of the androgens are independent of epithelial receptors. Rather it is the presence of

receptors on the stromal fibroblasts which are essential for epithelial differentiation (Cunha, 1984). Another example involves estrogen. Estrogen binds to stromal fibroblast receptors in the uterus and induces the maturation of the uterine epithelium (Taguchi et al., 1983). Thus the differentiation of several types of epithelia is under fibroblastic control, which in turn appears to be regulated by systemic hormones (Freshney, 1992).

As stated previously, mesodermal cells have a known influence on the maturation of epithelial cells (Cunha and Lung, 1979; Smith and Strickland, 1981; Simon-Assman et al., 1990). The mesodermal cells may produce soluble factors which influence hepatocyte differentiation but they may also influence hepatocyte differentiation through the production of the extracelluar matrix (ECM).

All liver cells except Pit cells (whose function is unknown) produce ECM components (Rojkind & Ponce-Noyola, 1982). For example, primary cultures of hepatocytes which maintain the ability to synthesize albumin are also able to produce collagen, glycosaminoglycans, laminin, and fibronectin. The ECM is composed of components generated by the cells associated with it, and therefore ECMs formed by different cell types (fibroblasts, epithelium, endothelium) will have different compositions (Freshney, 1992). The different components of the ECM include several insoluble factors, all of which favour cell attachment. Some

encourage cells to spread out while others are responsible for maintaining the cuboidal shape of cells which, in hepatocytes, is important for the maintenance of cell function (Ben-Ze'ev et al., 1985).

In the liver there are several collagens (types I, III, IV, and VI), some noncollagenous glycoproteins such as fibronectin and laminin, and heparin sulphate proteoglycans (Clement et al., 1986). The composition of the ECM may vary. For example, collagen is found in four major forms, but there may be up to twelve forms in total (Vuorio and Crombrugghe, 1990). Fibronectin (Enrich, et al., 1988) and laminin are fairly consistent in structure but the proteoglycans vary greatly. Proteoglycans are composed of proteins which are bound to four main types of glycosaminoglycans (GAGs): heparan sulphate, chondroitin sulphate, dermatan sulphate, and nonsulfated hyaluronic acid (Freshney, 1992). Within each group of GAG there is great variability in the molecular size, degree of branching, position and type of GAGs, etc. (Jalkamen, 1987).

The components of the ECM are found in various locations within the liver (reviewed in (Rojkind & Ponce-Noyola, 1982)). Collagen I is found in the liver capsule and in the stroma of large blood vessels. Type III collagen is found associated with type I collagen about large vessels, forms reticular fibres, forms a mesh inside the liver tissue, and is found inside the space of Disse in

association with Ito cell processes. Type IV collagen, fibronectin, and laminin all have a similar distribution in the liver. They are present in the basal lamina, around arteries, lymphatics, bile ducts, bile ductules, nerve endings, and between the endothelial lining of the sinusoids. Type V collagen is present throughout the liver but is concentrated at vessels. It is present at the sinusoidal surface of hepatocytes but is not present in sites containing true basal lamina such as the bile ducts and ductules.

The ECM is a complex environmental factor and as such is hard to manipulate as a whole. However there is some evidence that simple manipulation of the ECM leads to an extension of the differentiated functions of epithelia (Caron, 1990). Thus the ECM is an essential component in epithelial differentiation and may play a central role in the contact mediated effect of some cell-cell interactions (Simon-Assman et al., 1986). ECM components have also been found to influence hepatocyte growth in culture (Bissell et al., 1986; Bissell et al., 1987; Spray et al., 1987). Recently hepatocytes have been cultured between layers of collagen type I. This treatment restores hepatocyte differentiation and maintains high albumin production for an extended period of time (Dunn et al., 1989). Other advances have been made using complexes of hormones and biomatrix materials (Enat et al., 1984), but the main advancement has

been made with a laminin-rich gel called Matrigel (Bissell et al., 1987). Cells plated on this substrate retain their spherical shape and exhibit enhanced expression of several liver-specific functions, such as plasma protein production, and the production of drug and glycogen metabolism enzymes for a period of weeks (Guzelian et al., 1988). Matrigel is very rich in laminin but it also contains other extracelluar components such as collagen IV and heparan sulphate proteoglycans. It may also contain growth factors such as TGF-β. This heterogeneity makes it difficult to determine what factors are critical in maintaining the hepatocyte differentiated functions (Guguen-Guillouzo, 1992).

Like ECM components, cell adhesion is fundamentally important in morphogenesis and cytodifferentiation, and is especially important in the formation and maintenance of a polarized cellular phenotype (Stamatoglou et al., 1992). Between 12 and 24 hours in aggregate culture, liver cells have replaced weak intercellular bonds with stronger bonds which firmly bind the aggregates together (Landry et al., 1985). This aggregation of cells results in a change in the shape of the cells which may allow greater cell-cell interactions via cell junctions or soluble signals, and/or cause the cells to accumulate and deposit a more natural ECM than are seen in monolayer cultures. This aspect of cell culture and its importance in hepatocyte polarization is discussed further in chapter two.

Summary

In this chapter I have described early liver development, outlined the histology of the liver, given a brief introduction to previous methods of liver cell culture, and outlined factors which may be involved in the polarization of hepatocytes. In the next chapter I present a paper which shows that FCS contains an active inhibitor of hepatocyte polarization in monolayer culture, and that cell-cell interactions mediated by E-cadherin are needed for the formation and maintenance of extended networks of BC, but not for the expression of BC-specific antigens or for the maintenance of a polarized distribution of those antigens.

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CHAPTER II

Effects of fetal calf serum and disruption of cadherin function on the formation of bile canaliculi between hepatocytes

Introduction

The liver begins to form in the developing chicken embryo during the second day of incubation (Kingsbury, et al., 1956). The bile canalicular network, which is necessary for collection and transport of bile, only begins to develop on day 4 and does not spread completely throughout the liver until day 7 (Gallin & Sanders, 1992). This network is formed by the polarization of hepatocytes, creating two structurally and functionally distinct membrane domains: the basal-lateral domain and the apical or bile canalicular domain (Hubbard, et al., 1983; Hubbard, et al., 1985). The apical and basal-lateral membrane domains are generated by an asymmetrical targeting of membrane-bound cellular components and are maintained by tight junctions which present a physical barrier to the movement of membrane-bound constituents into inappropriate membrane domains (Hubbard, et al., 1983).

The factors that are responsible for hepatocyte polarization and the resultant formation and maintenance of BC are largely unknown. However, cell shape (Ben-Ze'ev,

1991; Ingber, 1991), interactions with extracellular matrix materials such as collagen and fibronectin (Mustat, et al., 1993), vascularization of the liver tissue (providing soluble cues such as growth factors), and communication with other cells via gap junctions (Meyer & Overton, 1983) or cell surface molecules such as cadherins may all play roles.

Attempts to use primary cultures of hepatocytes to study cell polarization have been hampered by the rapid dedifferentiation of the cells. Expression of BC-specific antigens in monolayer cultures of embryonic hepatocytes is transient (less than 3 days) when the hepatocytes are cultured in medium with 10% fetal calf serum (FCS)(Gallin & Sanders, 1992). Other investigators have found that a culture medium supplemented with insulin, dexamethasone, and ornithine allows rat hepatocyte monolayers to maintain a differentiated state for at least 3 days, as evaluated by morphology, mRNA transcription, and translation (Marceau, et al., 1984). I used DMEM supplemented with insulin, dexamethasone, and ornithine to extend to 7 days the time during which 14-day embryonic chick hepatocytes, grown in monolayer culture, maintain a polarized distribution of BC-specific antigens. A unique set of antibodies was used to document changes in the distribution of BC-specific antigens on the surface of hepatocytes over time in various media. Using this system I have determined that FCS contains a soluble inhibitor of hepatocyte polarization.

I also studied the development and maintenance of BC in hepatocytes cultured as aggregates in suspension. Hepatocyte aggregates more closely mimic *in vivo* conditions than do monolayer cultures (Moscona, 1961; Landry, et al., 1985). In aggregates, three-dimensional interactions between cells may support a more normal production of the ECM and allow higher levels of homologous and heterologous cell-cell contacts than are seen in monolayer cultures (Landry, et al., 1985). Homologous cell-cell contact in hepatocytes is mediated in part by E-cadherin. Thus, increased E-cadherin mediated membrane contact may be a factor in the formation and maintenance of BC.

I addressed the importance of cell-cell interactions on hepatocyte polarization by disrupting cell-cell adhesion with anti-E-cadherin Fab' fragments. As with other cell adhesion molecules, homophilic binding of liver cells via E-cadherin (originally called L-CAM in the chicken (Gallin, et al., 1983)) may alter the responses of cells to local signals through alteration of intercellular binding strength, the intercellular spacing, or cytoskeletal dynamics which might affect cell shape and signal processing (Edelman & Crossin, 1991). The cytoplasmic domain of E-cadherin interacts with cytoplasmic proteins called catenins (Ozawa, 1990) which are involved in several cell signalling pathways (Su, et al., 1993; Puffer, et al., 1992; Mc Crea, et al., 1993) and E-cadherin has been found to

function in the polarization of MDCK cells (Nelson, et al., 1990). Thus the adhesive nature of E-cadherin and its possible role in signal transduction suggest a role in the formation and maintenance of BC.

To determine if E-cadherin-mediated cell-cell adhesion is necessary for the formation and maintenance of BC I treated hepatocyte monolayers with anti-E-cadherin Fab' fragments. Using immunofluorescence and TEM analysis I were able to document changes in BC structure.

In summary, I have analyzed the inhibitory effects of fetal calf serum on hepatocyte polarity. Then, using culture conditions that were optimized for maintaining polarity, I evaluated the role of cadherin-mediated adhesion in the formation of bile canaliculi.

Materials and Methods

Preparation of liver cells: Under sterile conditions, 14-day chick liver rudiments were excised and digested with collagenase and crude trypsin, and a single-cell suspension was prepared as previously described (Bertolotti, et al., 1980).

Media: Dulbecco's Minimal Essential Medium with 10% fetal calf serum, and 1 µg/ml gentamicin (DF) was used for serum enriched cultures. Dulbecco's Minimal Essential Medium with Pen/Strep (penicillin 10000 U/ml and streptomycin 10 mg/ml)

(D), D plus $0.2X10^3$ U/ml insulin, 67.4 mg/L ornithine, and 10 μM dexamethasone (DIOD), and DIOD plus 0.5% fatty acid free BSA (DB) (Marceau, et al., 1984) were used as serum unsupplemented media.

Preparation of monolayer cultures: 1.7×10^6 liver cells in 0.5 ml of medium were cultured on a flame-sterilized, round, 10 mm coverslip in a 24 well Falcon tissue culture plate. Cultures were incubated in a 10% CO_2 incubator at 37°C. Medium was exchanged every two days for 0.5 ml of fresh medium.

Preparation of aggregate cultures: 3.4×10^7 liver cells were cultured in 10 ml of DF, or DIOD supplemented with 25 mM HEPES buffer, in silane coated, sterile 50 ml Erlenmeyer flasks. Cultures were exposed to 10% CO_2 for one hour, sealed, and then incubated in a 37°C water bath on a plate rotating at 70 r.p.m. with a stroke diameter of 1 cm. The medium was exchanged for fresh medium and regassed every second day of incubation.

Antibody Preparation: BC-specific monoclonal antibodies were prepared as described previously (Gallin & Sanders, 1992). Goat anti-E-cadherin (chicken) was prepared as described previously in rabbits (Gallin, et al., 1983). Fab' fragments of goat anti-E-cadherin, and non-specific

goat antibodies were prepared by pepsin digestion as previously described (Acheson & Gallin, 1992). All were prepared as 6 mg/ml stocks and were used at a concentration of 300 µg/ml.

Fixation and Staining:

Immunofluorescence: Monolayers and aggregates were fixed in -20°C methanol for 10 minutes. Immediately prior to staining, the cells were washed 5 X 10 min in TBS (50 mM Tris-HCl pH 8.0, 150 mM of NaCl)and then blocked with 10% BSA in TBS overnight at 4°C. Samples were incubated with 0.5 ml of undiluted hybridoma culture supernatant at 4°C for 2 hours. The samples were washed 5 X 1 h in TBS prior to the addition of FITC rabbit-anti-mouse secondary antibody, diluted 1:200 in 10% BSA in TBS. The samples were incubated at 4°C overnight, washed 5 X 1 h in TBS, counter-stained with 1% Evan's blue for 2 hours, and mounted on glass slides with Mowiol -DABCO (Osborn & Weber, 1982).

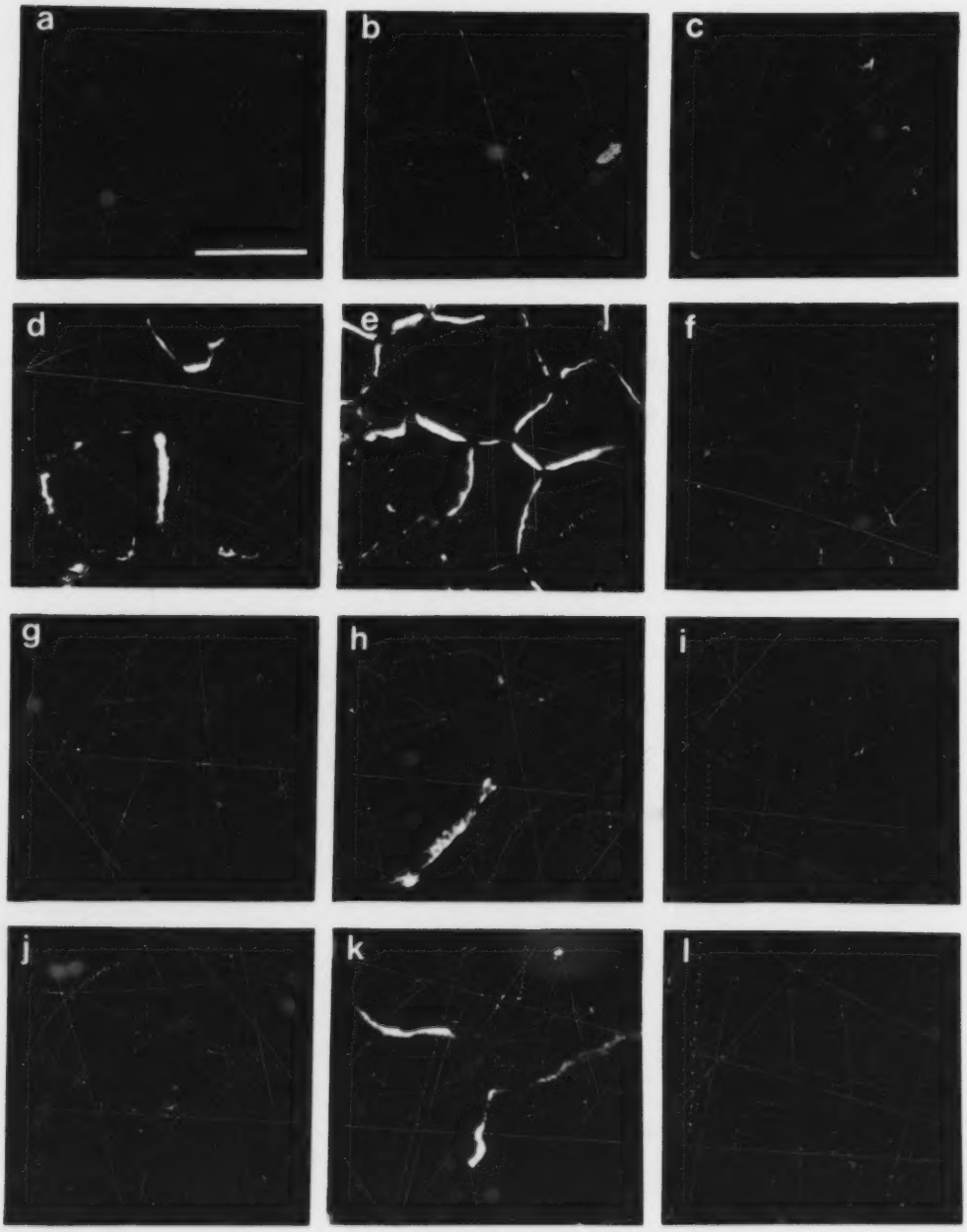
Transmission Electron Microscopy: Monolayers were fixed in 2% glutaraldehyde in 0.02 M phosphate buffer (PB) (0.39 g of $NaH_2PO_4.H_2O$ and 1.93 g of $Na_2HPO_4.7H_2O$ dissolved in 500 ml of water, pH 7.2) for 1 h, washed 2 X 15 min in PB, then secondarily fixed in 2% osmium tetroxide diluted in PB for 1 h. The tissue was washed for 2 X 15 min in PB and

dehydrated in a series of 15 min ethanol washes. Samples were then exchanged in propylene oxide for 3 X 15 min, placed for 2 hours in 25% Epon in propylene oxide, transferred to 50% Epon in propylene oxide and incubated overnight at 4°C. The monolayers were then placed cells up on clean glass slides and 100% Epon was layered on top of the cells. The cultures were let stand at room temperature for 10 min and then microfuge tubes full of Epon were placed on top of the monolayers. These samples were then hardened at 60°C for 48 hours. Monolayers were repeatedly immersed in liquid nitrogen for several seconds to separate the embedded hepatocyte monolayers from their glass substrate. Silver-gold sections were cut with a diamond knife and placed on acetone-washed grids. Grids were stained with saturated uranyl acetate in 50% ethanol for 10 min, washed with 50% ethanol, deionized water, and then dried on filter paper. The secondary stain was Reynolds' lead citrate for 30 min. Then the grids were washed with alternating applications of 0.02 N NaOH and deionized water.

Results

Monolayer cultures lose all BC-specific antigen expression by 3 days when cultured in a medium supplemented with FCS (Figure II-1 and ref. (Gallin & Sanders, 1992)). However, monolayer cultures grown in DMEM alone were able to maintain their BC-specific antigen expression for 5 days

Figure II-1. Monolayer cultures of hepatocytes grown in DF for 3 days (a, d, g, j), DMEM for 5 days (b, e, h, k), or DIOD with 10% FCS for 3 days (c, f, i, l). The cultures were stained with mAb 3B8 anti-dog-E-cadherin (a, b, c) as a negative control, mAb 7C5 anti-chicken-E-cadherin (d, e, f), and mAb 1H6 (g, h, i) or mAb 8G7-G7 (j, k, l) which both bind antigens localized in BC. DF and DIOD with 10% FCS treated monolayers have lost their BC-specific antigen expression within 3 days of culture initiation while the cultures grown in DMEM maintain BC-specific antigen expression for 5 days. Scale bar = 35 µm.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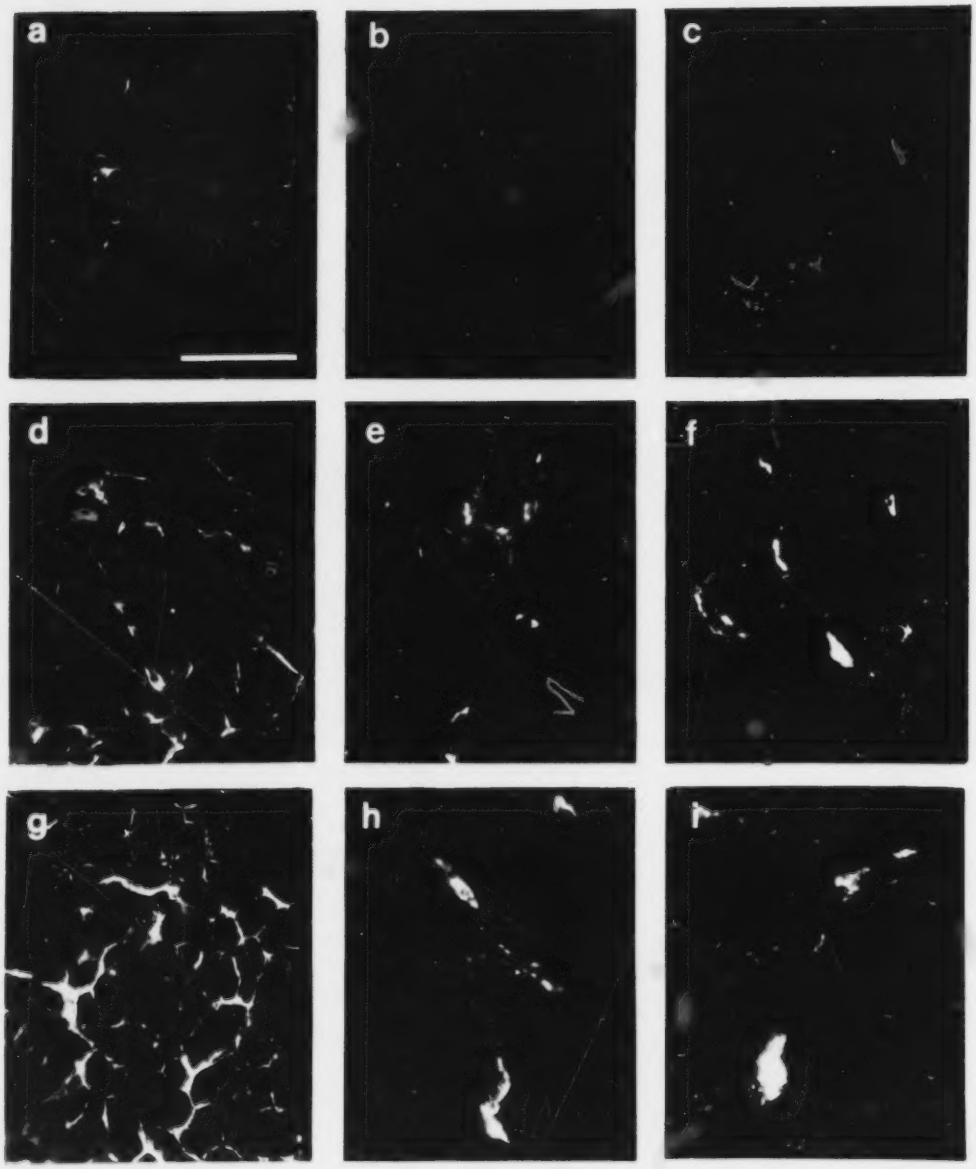
(Figure II-1). Thus FCS appears to have an inhibitory effect on hepatocyte polarization. Media containing dexamethasone, insulin, and ornithine have been used to support the survival of differentiated suckling rat hepatocytes (Marceau, et al., 1984; Kawahara & French, 1990). Therefore I cultured 14-day chick hepatocyte monolayers in various serum-free media to determine if these additives will prolong cell polarization. Media used include: DMEM alone, DIOD, and DB (Marceau, et al., 1984). I found that DIOD
and DB supported BC formation and antigen expression for 7-10 days of culture (data not shown). There was no observable difference between cultures grown with or without fatty acid free BSA. Therefore I used DIOD in subsequent cultures.

I then confirmed that the loss of BC-specific antigen expression seen in FCS treated cultures was due to an active inhibitor of antigen expression by culturing hepatocytes in DIOD with 10% FCS. The loss of BC-specific antigen expression in these cultures was identical to that seen in cultures grown in DF. In both cases the expression of BC-specific antigens was rapidly diminished and undetectable by the third day of incubation (Figure II-1). Thus, the loss of antigen expression caused by FCS is due to a factor or factors that actively inhibit BC-specific antigen expression in hepatocytes.

I next determined whether the loss of expression seen in FCS-treated monolayer cultures could be reversed. Figure II-2 shows that the effects of FCS on the polarization of hepatocytes were reversed when the cultures are switched to DIOD. The cells regained the expression of the five BC-specific antigens I assayed for (1H6 and 8G7-G7 are shown in the figure) and these antigens were expressed in the lumen of long BC networks. This recovery is even possible after 3 days in FCS-supplemented medium, when no BC-specific antigen expression is visible in hepatocyte

Figure II-2. Hepatocyte monolayers after culture in DF for 5 days (a, b, c), DF for 3 days and then DIOD for 2 days (d, e, f), or DF for 3 days and then DIOD for 4 days (g, h, i). The cultures were stained with mAb 7C5 anti-chicken-E-cadherin (a, d, g), and mAb 1H6 (b, e, h) or mAb 8G7-G7 (c, f, i) antibodies which bind antigens localized in the BC. All BC-specific antigen expression is lost in the culture exposed only to DF for 5 days or for 3 days (see Fig. 1). However, the culture which was exposed to FCS for 3 days and DIOD for 2 days has regained its BC-specific antigen expression. The culture exposed to DF for 3 days then to DIOD for 4 days shows that the recovered BC form longer networks and are expressed for as long as those in cultures grown only in DIOD. Scale bar = 73 μm.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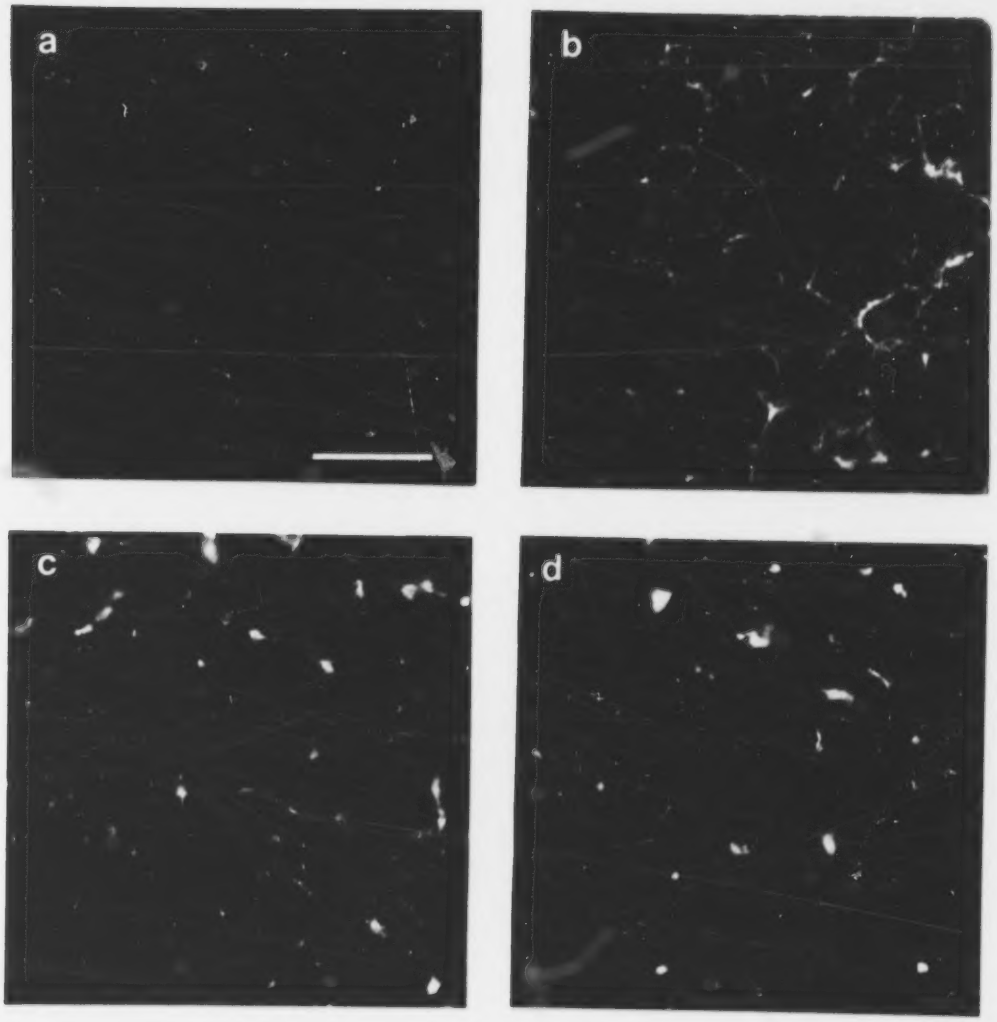
monolayers (Figure II-1). Furthermore, BC-specific antigens are present at 7 days of culture in hepatocyte monolayers exposed to DF for 3 days then switched to DIOD (Figure II-2). This expression is identical to that seen in cultures grown only in DIOD.

Aggregate cultures grown in DF (Figure II-3) or DIOD (data not shown) are able to maintain BC-specific antigen expression for 10-12 days. This indicates that aggregates are resistant to the factor(s) in FCS which cause the loss of BC-specific antigen expression in monolayers and that aggregate culture provides conditions more conducive to maintaining polarization than does monolayer culture. The ability of aggregate cultures to maintain BC-specific antigen expression may be due to a number of factors, including the production and appropriate deposition of the ECM, the shape of the hepatocytes, and the amount of cell-cell contact. Aggregates may also exclude the de-differentiating factor in FCS from the interior of aggregates.

Aggregates of liver cells are able to form a more normal ECM, both in composition and structure, than that seen in monolayer cultures (Landry, et al., 1985). The hepatocytes are able to interact with the ECM in three dimensions and this may influence the ability of the hepatocytes to form and maintain BC. Homologous and heterologous cell-cell interactions also may be required for

Figure II-3. Hepatocyte aggregates cultured in DF for 12 days. Cultures were stained with mAb 3B8 anti-dog-E-cadherin (a) as a negative control, mAb 7C5 anti-chicken-E-cadherin (b), and mAb 1H6 (c) or mAb 8G7-G7 (d) which both bind antigens localized in BC. E-cadherin is present uniformly on the cell surfaces, the BC antigens are present in a distinct three-dimensional network of canaliculi. Scale bar = 73 μm.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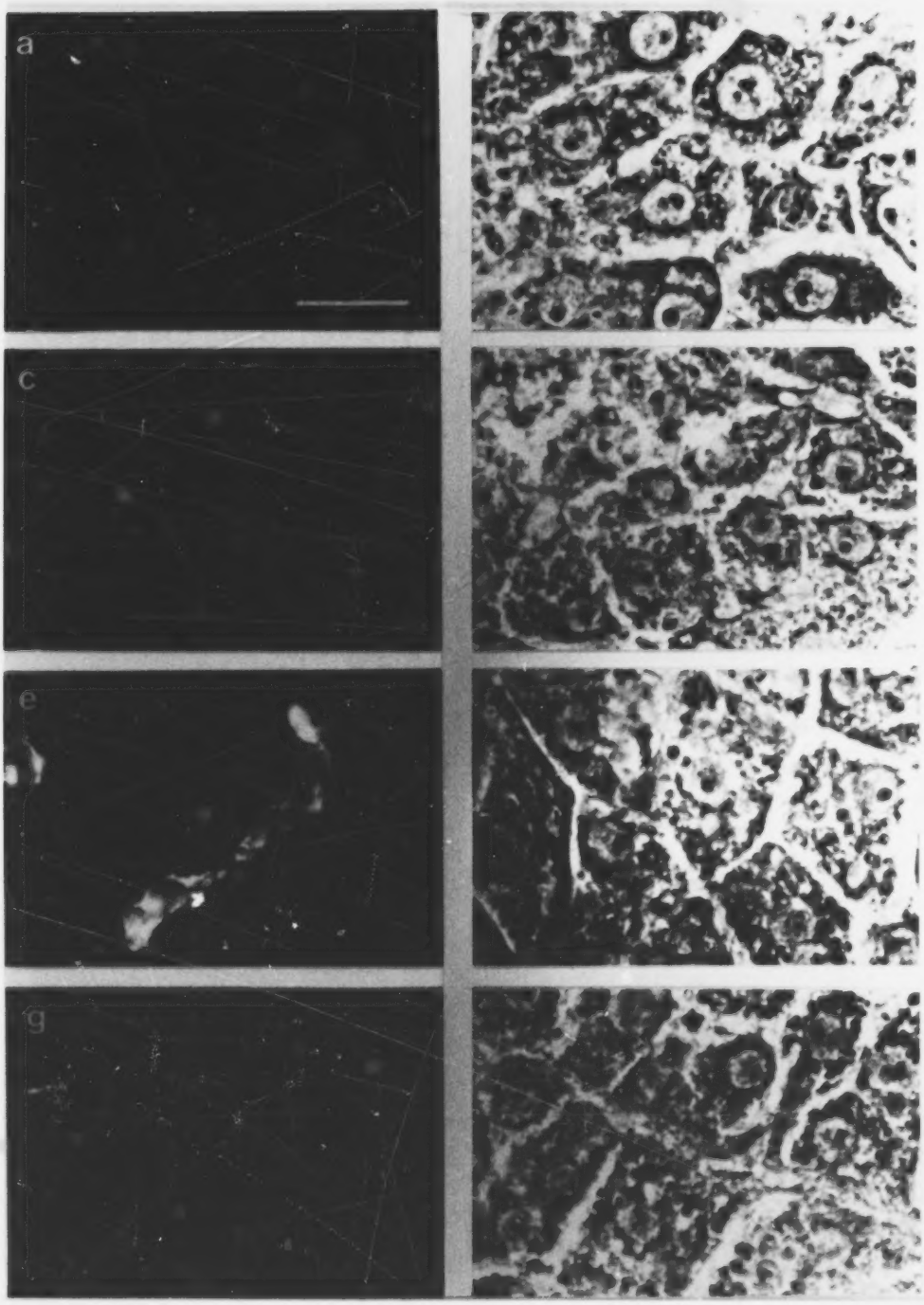
d

BC formation. In aggregate cultures and *in vivo*, hepatocytes have more extensive lateral cell-cell contacts than in monolayers because of the differences in cell shape and the presence of other hepatocytes in all three dimensions, not just the two dimensions of a monolayer culture. Also, hepatocytes in aggregate culture or *in vivo* do not have a large, free, non-apical surface, as they do in monolayer culture. Thus, another factor to be considered in the formation and maintenance of BC is the extent of E-cadherin mediated cell-cell interactions.

If extensive cell-cell interactions mediated by E-cadherin are vital to the formation and maintenance of BC and the decreased stability of BC-specific antigen expression seen in monolayers is due to sub-optimal E-cadherin mediated cell-cell adhesion, then complete disruption of E-cadherin mediated cell-cell adhesion should prevent the expression of BC-specific antigens and the polarization of hepatocytes. Antibodies to E-cadherin were used to perturb the adhesion and compaction of hepatocytes grown as monolayers. I compared cultures of hepatocytes grown for 5 days in DIOD alone, in DIOD containing 0.3 mg/ml Fab' fragments of antibodies to E-cadherin, and in DIOD containing 0.3 mg/ml of nonspecific goat Fab'. There was no difference between the 5-day no Fab' or non-immune goat Fab' treated cultures: both consisted of tightly associated cells with extensive BC networks (Figure II-4).

Figure II-4. Fluorescence (a, c, e, g) and phase (b, d, f, h) micrographs of control nonspecific goat Fab' treated 5-day monolayers. The monolayers have been stained with mAb 3B8 anti-dog-E-cadherin (a) as a negative control, mAb 7C5 anti-chicken E-cadherin (c), and mAb 1H6 (e) or mAb 8G7-G7 (g) antibodies which bind antigens localized in the BC. Scale bar = 36 μm.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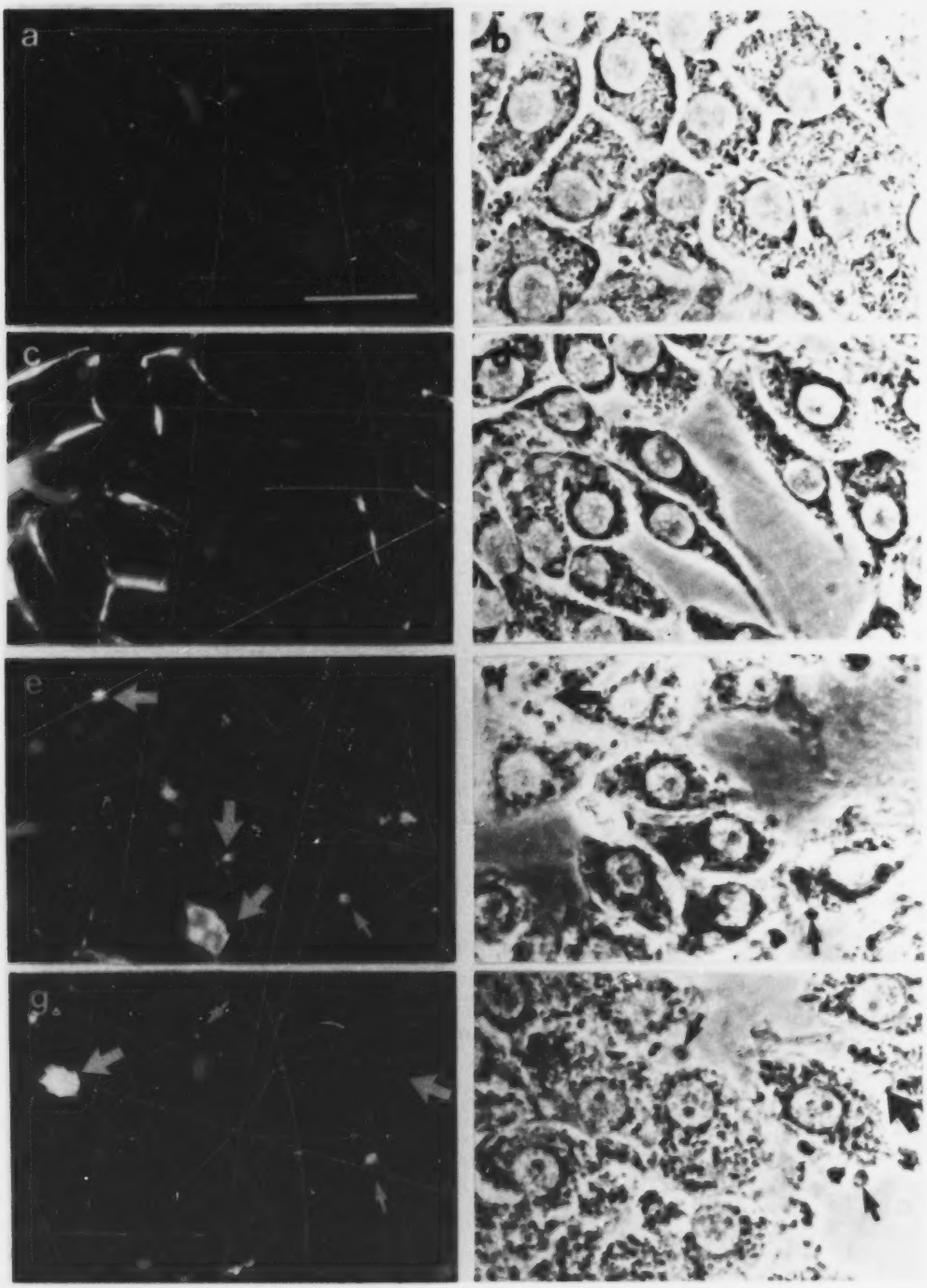
g

Figure II-5. Fluorescence (a, c, e, g) and phase (b d, f, h) micrographs of Fab' fragment anti-E-cadherin treated, 5-day monolayers. The monolayers have been stained with mAb 3B8 anti-dog-E-cadherin (a) as a negative control, mAb 7C5 anti-chicken E-cadherin (c), and mAb IH6 (e) or mAb 8G7-G7 (g) antibodies which bind antigens localized in the BC. The BC-specific antigens are now distributed at high concentrations in small cysts (large arrows) or in cytoplasts (small arrows) and at low concentrations on the rest of the cell surface. Scale bar = 35 μm.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The anti-E-cadherin treated cultures showed loose cell-cell contact and a disruption of the BC network (Figure 5). The BC antigens were expressed in anti-E-cadherin treated cells, but were concentrated in small cysts between cells and on cytoplasts (anucleate cell fragments) found on free cell surfaces. They were also observed at low uniform concentrations over the whole cell surface.

Antibody-treated monolayers were also examined by TEM (Figure 6). The hepatocytes treated with anti-E-cadherin antibodies (Figure 6 c) had much smaller BC cyst lumens than the lumenae of the ramified BC seen in untreated hepatocytes or hepatocytes treated with Goat Fab' (Figure 6 a and b). Anti-E-cadherin-treated and control cultures both had junctional complex-like features and no detectable difference was seen in the space between adjacent cell membranes in areas of high cell density. However, in areas of low cell density the anti-E-cadherin-treated cells were not closely associated and had large intercellular spaces. Figure 6 d and e show membrane-bounded structures associated with the surface of anti-E-cadherin treated hepatocytes. These structures contain few organelles other than vesicles and a few mitochondria and appear to have formed from pinching off of the cell plasma membrane. The position, and size of these structures indicate that they are probably the cytoplasts seen in fluorescence micrographs.

Figure II-6. TEM analysis of untreated (a), control nonspecific goat Fab' treated (b), Fab' fragment anti-E-cadherin treated (c-e) 5-day monolayers. Small arrows indicate the microvilli, asterisks indicate the cytoplast-like structures, stars indicate cells, and large arrows indicate the junctional complex-like structures. Scale bars in a, b, d, and e = 1400 nm. The scale bar in figure c = 350 nm.

a
b

Discussion

Polarization of hepatocytes is needed for the liver to function; thus, hepatocyte cultures used to study physiologically relevant events in liver cells must polarize and maintain that polarization. The factors responsible for hepatocyte polarization are largely unknown. I have defined some of the factors that affect hepatocyte polarization in primary culture.

Early attempts at culturing purified hepatocytes were unsuccessful in maintaining the cells in a differentiated state for more than a few days (Chapman, et al., 1973; Guguen, et al., 1975; Guguen-Guillouzo & Guillouzo, 1983). This lack of success was thought to be due to an absence of appropriate nutrients in the culture medium, a requirement for an appropriate extracellular matrix for attachment and growth of the cells (Reid & Rojkind, 1979; Reid, et al., 1980), the absence of other cells with which the hepatocytes interact *in vivo* (Guguen-Guillouzo & Guillouzo, 1983), or down regulation of liver-specific mRNA transcription (Clayton & Darnell, 1983) or translation, ultimately caused by alteration of the cytoskeleton as cells plate into two-dimensional monolayers from three-dimensional organs (Ben-Ze'ev, 1991).

Fetal calf serum supplemented media are known to be detrimental to hepatocyte proliferation and expression of liver-specific functions in culture (Enat, et al., 1984).

However, FCS supplemented media will support the polarization of hepatocytes for 2 days in monolayer culture (Gallin & Sanders, 1992; Wanson, et al., 1977). By 3-4 days the cultured hepatocytes depolarize, losing their BC and differentiated functions (Gallin & Sanders, 1992; Wanson, et al., 1977) unless supplements such as dexamethasone and insulin are added to the culture media (Marceau, et al., 1984) or ECM components such as collagen are plated on top of the cells (Dunn, et al., 1992).

The preservation of hepatocyte activity seen with the addition of dexamethasone (or other glucocorticoids) and insulin may be linked to the preservation of cell shape through the synthesis of cytoskeletal elements such as cytokeratin (Marceau, et al., 1984). Dexamethasone enhances the synthesis of a 55,000 Mr, 49,000 Mr, and 51,000 Mr cytokeratin components depending on the concentration of dexamethasone used and the initial seeding density of the hepatocytes (Marceau, et al., 1984). Under conditions of increased cytokeratin production, differentiated functions such as albumin production are enhanced (Marceau, et al., 1984). However, the mechanism by which cytokeratin production influences the differentiated functions of hepatocytes is unknown. Dexamethasone is also involved in inhibiting the actions of hepatocyte growth factor (Matsumoto, et al., 1992), enhancing fibronectin synthesis (Odenthal, et al., 1992), and preserving and promoting gap

junctional communication between hepatocytes (Ruch & Klaunig, 1992). I used a medium supplemented with insulin, dexamethasone, and ornithine to culture 14-day chick hepatocytes and found that hepatocyte monolayers were able to maintain BC for at least 7 days.

Monolayer cultures that had been maintained for 3 days in DF and then for several days in DIOD were able to reform BC. Therefore DF does not permanently stop the expression of BC-specific proteins. However, as seen in the DIOD with 10% FCS treated cultures, the inhibitory effects of FCS are able to override the stimulatory effects of ornithine, insulin, and dexamethasone. Thus, the loss of polarity caused by FCS appears to be due to some factor that actively inhibits the maintenance of polarization and appropriate cytodifferentiation in hepatocytes in a reversible manner, and not to a lack of some essential factor in the culture medium.

Aggregate cultures grown in DF or DIOD are able to maintain BC-specific antigen expression longer than monolayer cultures. Aggregates may be resistant to the depolarizing effects of FCS due to their extensive lateral cell-cell contacts and the low ratio of surface area to total aggregate volume, which would preclude penetration into the central regions of the aggregate. However, exclusion of FCS from hepatocyte aggregates does not explain why aggregate cultures maintain their polarization longer

than monolayer cultures when grown in DIOD or DMEM alone. Previous studies on aggregate cultures of hepatocytes (Moscona, 1961; Landry, et al., 1985) have shown that cells may need a three-dimensional cyto-architecture, as seen *in vivo*, for optimal function (reviewed in (Landry & Freyer, 1984)). Under these conditions cells survive and differentiated functions are maintained in the absence of added hormones or serum factors. Possible explanations for this are the production and deposition of an appropriate ECM and/or an appropriate pattern of cell-cell contact, both dependent on three-dimensional homotypic and heterotypic cell-cell interactions (Landry, et al., 1985).

Cell adhesion is important in morphogenesis, cytodifferentiation, and in the establishment and maintenance of cell polarity in epithelial cells (Edelman, 1986; Ekblom, et al., 1986; Simons & Fuller, 1985; Takeichi, 1991; Trelstad, 1984; Wiley, et al., 1990). Specific cell-cell and cell-substratum contacts are needed to initiate the development of epithelial polarity (Ekblom, et al., 1986; Klein, et al., 1988) by causing the expression of new gene products (Ben-Ze'ev, 1991), and the redistribution of constitutively expressed proteins (Simons & Fuller, 1985; Rodriguez-Boulan & Nelson, 1989). E-cadherin is important in the compaction (Hyafil, et al., 1980) of cells and the initial formation of intercellular junctions (Gumbiner, et al., 1988).

Aggregation in liver cells is a two phase process. First single cells aggregate loosely, due to interactions between E-cadherin molecules on the cell surface and interactions between E-cadherin and the cytoskeleton (Takeichi, 1991). The second phase of liver aggregation involves compaction of the aggregated cells. This compaction phase is dependent on the presence of calcium, protein and RNA synthesis, and the production of energy (Landry, et al., 1985). Cells in aggregate culture have extensive cell-cell contact and little free apical surface. Cells in monolayers have limited cell contact around the cell margins and a free non-apical surface. Thus, the more rapid loss of polarity in monolayer cultures may result from the two-dimensional environment imposed on the hepatocytes (Folkman & Greenspan, 1975). If cell-cell interactions are vital to the maintenance of BC and the decreased stability in monolayer cultures is due to less E-cadherin-mediated adhesion than in aggregates, then complete disruption of E-cadherin mediated cell-cell adhesion should prevent the expression and polarization of BC-specific antigens. Thus I used anti-E-cadherin antibodies to determine if disruption of cadherin mediated cell-cell interactions would affect hepatocyte polarization or the maintenance of BC.

I have shown, in monolayers, that perturbation of cell-cell adhesion with antibodies to E-cadherin causes a decrease in the long range organization of the BC-like

structures found in 14-day chick hepatocytes, but not a loss of BC-specific protein expression. I also found a low concentration of BC-specific antigens distributed uniformly over the surface of the cells. These results are not due to non-specific effects of the antibody treatment because identical treatment of hepatocyte monolayers with nonspecific goat Fab' antibodies has no effect on the polarization of the cells. The cysts between cells and cytoplasts on the surface of cells which are seen to contain the BC-specific antigens may have resulted from incomplete disruption of cell-cell adhesion by anti-E-cadherin Fab' fragments. However, I found that 300 µg/ml of anti-E-cadherin gives a maximal response and doubling the concentration of antibody to 0.6 mg/ml does not change the distribution of BC-specific antigens. Thus, some other factor may be responsible for the integrity of the cysts and surface cytoplasts seen in anti-E-cadherin treated cultures. Citi (Citi, 1992) has shown that some protein kinase inhibitors prevent junctional dissociation in MDCK cells exposed to a low calcium medium which disrupts E-cadherin function. Thus, protein kinases, and possibly other intracellular factors, are involved in the maintenance of junctional complexes independent of E-cadherin.

The BC-specific antigens found in localized regions of the hepatocyte plasma membrane in anti-E-cadherin treated cells may be prevented from diffusing over the cell surface

by a number of factors. Tight junctions may prevent the antigens from exiting membranous cysts or cytoplasts. The antigens may also be associated with cytoskeletal components which prevent them from dispersing.

There are several reasons why treatment of hepatocytes with anti-E-cadherin antibodies might cause a disruption of long range networks of BC. The simplest explanation is purely mechanical; if cells can not form strong contacts, they can not form co-ordinated networks. Another explanation is suggested by experiments using anti-A-CAM (anti-N-cadherin) antibodies to perturb Novikoff (rat hepatoma adapted for growth in suspension culture) cells (Meyer, et al., 1992). These antibodies disrupt gap junction assembly and inhibit adherens junction formation. Anti-E-cadherin treatment of hepatocytes may have similar effects. Uncoupling of the hepatocytes by disruption of signal transduction via gap junctions could be responsible for the decrease in hepatocyte BC network length seen in monolayer cultures. The limited low level of uniform surface staining seen in anti-E-cadherin treated hepatocytes may be caused by partial disruption of tight and adherens junctions. TEM photomicrographs of anti-E-cadherin treated hepatocytes show junctional complex-like structures located around BC cysts. However, these complexes may be missing key components or the components may not be functioning optimally.

A decrease in the ability of hepatocytes to communicate and form long range BC networks may also hinder protein trafficking pathways. After synthesis the BC-specific proteins are transported to the basal-lateral membrane domain and then are transported into the apical domain of a hepatocyte (Bartles, et al., 1987). If the transport from the basal-lateral into the apical domain is inhibited the proteins are stranded on the basal-lateral domain and this could account for hepatocyte surface staining.

In summary, our results indicate that a FCS-free culture medium will prolong the presence of BC in monolayer cultures by eliminating the active depolarizing effects of FCS. Monolayers of hepatocytes maintain their polarization longest (7-10 days) when cultured in DIOD indicating that insulin, dexamethasone, and ornithine prolong the polarized expression of BC-specific antigens. Aggregates of hepatocytes are not affected by the depolarizing factor(s) in FCS and are able to maintain BC in DF and DIOD for 10-12 days, probably because of the increased cell-cell contact of aggregate cultured cells, the production and deposition of an appropriate ECM, or the shape of the cells. I also found that cell-cell adhesion is important for the formation and maintenance of extended networks of BC in monolayer cultures of chick hepatocytes. Treatment of hepatocytes with anti-E-cadherin Fab' fragments caused the formation of small BC cysts between cells, cytoplasts containing BC-specific

antigens on free cell surfaces, and a low level uniform distribution of BC-specific antigens over the surface of hepatocytes. Thus, cadherin mediated cell-cell interactions influence the ability of hepatocytes to form long range organizations of canaliculi and may play a role in the targeting of some BC-specific proteins. However, the expression of BC-specific antigens in 5-day and 7-day anti-E-cadherin-treated hepatocytes indicates that extensive cell-cell interactions are not required for differentiation of bile canaliculi.

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Chapter III

Discussion and Conclusions

The liver of a chicken forms from an invasion of the ductus venosus by diverticula which arise from the small intestine and branch repeatedly to form a system of hepatic cords and sinusoids. These cords and sinusoids allow the liver to interface the circulatory system and the digestive system, and are necessary for the liver to function. Hepatic cords are formed by the differentiation of hepatocytes and the formation of apical and basolateral domains in hepatocytes. These domains are delimitated by junctional complexes. One component of junctional complexes are the tight junctions which prevent the migration of domain-specific constituents into inappropriate domains, and allow hepatocytes to vectorially uptake matter from the sinusoids, process it, and excrete materials into the biliary transport system. The finest tubes in the biliary transport system are formed by the linkage of the apical domains of hepatocytes in a cord and are called bile canaliculi (BC). The factors responsible for the differentiation of hepatocytes and the formation of the network of BC are largely unknown. However, cell-cell interactions and the production of an appropriate ECM may play a role.

Hepatocyte monolayers are a model system for the analysis of common and liver-specific gene regulation, for

studying liver diseases, and are a tool for measuring the hepatotoxicity and metabolism of drugs (Guguen-Guillouzo, 1992). To be useful for these purposes the hepatocyte monolayers must maintain a polarized phenotype.

Pure hepatocyte populations can be maintained for about one week as monolayers of non-replicating cells. The cells form trabeculae, and within one day form bile canaliculi. The freshly isolated hepatocytes have functional capacities similar to those found *in vivo* (Clayton and Darnell 1983). However, the ability of the hepatocytes to perform liver-specific functions is reduced within 3 days of culture initiation (Gallin and Sanders, 1992; Guguen-Guillouzo and Guillouzo 1983; Sirica, et al. 1979; Steward et al. 1985) possibly due to a blockage of liver-specific mRNA transcription (Clayton and Darnell 1983). Additives such as insulin, dexamethasone, or ECM components prolong the expression of differentiated hepatocyte gene expression, although the mechanism(s) of action are currently unclear.

I found that treatment of hepatocyte monolayers with ornithine, insulin, and dexamethasone extends the period of time in which the hepatocytes express BC-specific antigens to over 7 days. This contrasts to the more rapid de-differentiation seen in FCS treated cultures (3 days) or DMEM alone (5 days). The antigens were found in a polarized distribution, located in extended networks of BC-like structures. Whether this prolongation of BC-specific

antigen expression is due to increased transcription of mRNA, stabilization of existing mRNA, or to increased translation of mRNA is currently unknown. However, previous work indicates that glucocorticoids and insulin help to stabilize liver-specific mRNAs (Jefferson, et al., 1984).

An alternative view of de-differentiation involves an overgrowth of the culture by cells such as stromal fibroblasts or undifferentiated epithelial cells (Freshney 1992). According to Freshney (1992) culture conditions either encourage cell proliferation or cell differentiation and the de-differentiation of epithelial cells seen by early experimenters was due to an overgrowth of the culture by un-differentiated cells. There is no re-differentiation of de-differentiated cells. Instead, manipulation of culture conditions causes the differentiation of previously un-differentiated cells. This does not apply to the data presented in my paper.

If it were true that de-differentiation of hepatocytes in monolayers is linked to an overgrowth of non-differentiated cells then the loss of hepatocyte polarization seen in FCS treated cultures is due to selective growth of early stage, non-differentiated hepatocytes or of mesenchymal cells (Enat, et al., 1984). However, I did not observe much cell proliferation in monolayer cultures of hepatocytes. Dunn et al., in 1992, showed that the de-differentiation seen in hepatocytes grown

on a monolayer of collagen can be reversed when a second layer of collagen is plated on top of the hepatocytes. I was able to get a similar result by switching hepatocyte culture media. Growth of hepatocyte monolayers in FCS for 3 days causes a complete loss of BC-specific antigen expression. However, I was able to get the cells to re-express BC-specific antigens by culturing the cells in DMEM supplemented with ornithine, insulin, and dexamethasone. This induction of differentiation in hepatocyte monolayers occurs rapidly, and consistently throughout the monolayer. The low rate of proliferation observed in hepatocyte monolayers indicates that the most probable cells to be expressing BC-specific antigens are those which had lost that expression when treated with FCS. Therefore, I conclude that de-differentiated hepatocytes are able to re-express their differentiated features.

I have found that cell-cell interactions mediated by E-cadherin are necessary for the formation of BC networks but are not needed for the expression of, or polarized distribution of, BC-specific antigens. Exposure of hepatocyte monolayers to anti-E-cadherin antibodies causes a decrease in the formation of long range networks of bile canaliculi which may be due to a decrease in the maintenance of junctional complex-like components. Imhof, et al., in 1983, treated MDCK cells with anti-Arc-1 (a canine E-cadherin homolog) and found that the cells loosened their

tight junctions, lost gap junctional communication, and became depolarized. My TEM micrographs show junctional complex-like structures in anti-E-cadherin treated hepatocytes; however, these structures may not contain all of the components necessary for the formation of extended BC networks or the components may not be functional.

The data presented in this thesis open a number of avenues for further investigation. If E-cadherin-mediated cell-cell interactions are not responsible for hepatocyte polarization is there some other cell surface molecules which may be important in hepatocyte polarization? Other molecules which may be involved in hepatocyte polarization may be other cadherins, other members of the immunoglobin superfamily, or integrins.

What changes does anti-E-cadherin treatment induce in the junctional complex? Previous investigators have found that anti-E-cadherin treatment causes a disruption of tight and gap junctions. The mode of action of these antibodies is currently unclear. However, binding of an antibody to the extracellular domain of E-cadherin may cause a conformational change in the cytoplasmic domain of the cadherin and this could influence how the cadherin interacts with the cytoskeleton. Furthermore, the antibody binding to the extracellular domain of E-cadherin could be preventing appropriate junctional complex formation by not allowing adjacent hepatocyte membranes to interact.

What is the de-differentiation factor in FCS? Hepatocyte growth factor is one candidate. It has been previously isolated from rabbit serum and is a powerful mitogenic agent (Naldini, et al., 1991). Other, common growth factors are transforming growth factor and epidermal growth factor.

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